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# *Propionibacterium acnes* and *Staphylococcus lugdunensis* Cause Pyogenic Osteomyelitis in an Intramedullary Nail Model in the Rabbit

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Running Head: *P. acnes* and *S. lugdunensis* implant-related osteomyelitis

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**Abstract**

*Propionibacterium acnes* and coagulase-negative staphylococci (CoNS) are opportunistic pathogens implicated in prosthetic joint and fracture fixation device-related infection. The purpose of this study was to determine if *P. acnes* and the CoNS species *Staphylococcus lugdunensis,* both isolated from 'aseptically failed' prosthetic hip joints, could cause osteomyelitis in an established implant-related osteomyelitis model in the rabbit, in the absence of implant material wear debris.

The histological features of *P. acnes* infection in the *in vivo* rabbit model were consistent with localized pyogenic osteomyelitis, and biofilm was present on all explanted IM nails. The animals displayed no outward signs of infection, such as swelling, lameness, weight loss, or elevated white cell count. In contrast, infection with *S. lugdunensis* resulted in histological features consistent with both pyogenic osteomyelitis and septic arthritis, and all *S. lugdunensis* animals displayed weight loss and an elevated white cell count despite biofilm detection in only two out six rabbits.

The differences in the histological and bacteriological profiles of the two species in this rabbit model of infection are reflective of their different clinical presentations; low-grade infection in the case of *P. acnes* and acute infection for *S. lugdunensis*. These results are especially important in relation to the growing recognition of chronic *P. acnes* biofilm infections in prosthetic joint failure and non-union of fracture fixations, which may be currently reported as 'aseptic' failure.

## INTRODUCTION

Implant-related osteomyelitis is a serious complication of joint replacement and fracture fixation procedures. In the majority of cases, these infections are caused by bacterial species that are either permanent or transient members of the human microbiota. Of the different bacterial species present on the human skin, only a small number are frequently associated with implant-related osteomyelitis. One of the most common causative microorganisms is *Staphylococcus aureus,* which causes an acute infection characterized by fever, localized swelling and osteolysis (54,56). In contrast, the exact role played by other 'less virulent' members of the skin microbiota in medical device-related infections, including the Gram-positive anaerobic-to-aerotolerant bacterium *Propionibacterium acnes* and coagulase-negative staphylococci (CoNS), has historically been less clear; these organisms were previously dismissed as contaminants when cultured from clinical specimens (9,11,39).

'Aseptic' implant failure is considered to result from periprosthetic osteolysis driven by wear debris arising from the implant materials (37). There is, however, a growing recognition that bacteria are an underestimated cause of sub-acute and chronic bone infections, 'aseptic' loosening of implants, and arthrodesis after osteosynthesis (10,15,50,57,59). In particular, *P. acnes* is being increasingly recognized as a primary pathogen in relation to prosthetic shoulder implant infection (23), but has also been linked to other medical device-related infections, including those associated with prosthetic hip joints and heart valves, as well as central nervous system shunts (16,19,63). In keeping with this pathogenic capacity, whole genome sequencing has revealed the presence of multiple putative virulence determinants including phase and antigenically variable adhesins, as well as multiple co-haemolysin CAMP factors (28). In contrast, *Staphylococcus lugdunensis* appears an unusually virulent CoNS (13,14) that has been linked to a wide range of acute infections, including those associated with prosthetic joints (47,51), osteomyelitis (33,62), septic arthritis (30), soft tissue infections (2) and infective endocarditis (24). Despite this, we still have relatively limited understanding of the role and incidence of *S. lugdunensis* in human disease, which may lead to an underestimation of its clinical relevance and, consequently, its importance in relation to implant-related osteomyelitis.

Although the pathogenic potential of *P. acnes and S. lugdunensis*, especially in relation to medical device infections is increasingly recognised, there has been a limited number of preclinical experimental studies investigating the ability of *P. acnes* or *S. lugdunensis* to cause implant-related osteomyelitis. The purpose of this study was, therefore, to observe the effects of *P. acnes and S.*

*lugdunensis* in an established rabbit model (32) of implant-related osteomyelitis, in the absence of implant material wear debris, and characterize the resultant infections with respect to histological and microbiological outcomes.

## MATERIALS AND METHODS

### *Ethics Statement*

The animal study was approved by the ethical committee of the canton Grisons in Switzerland (approval number 06/2008). The animal care and use protocol used for this study adheres to the Swiss animal protection law and its regulations. The bacterial isolates used in this study were obtained from adult patients who provided informed, written consent to participate in a clinical study approved by the Faculty of Medicine Research Ethics Committee at Queen's University, Belfast. The *P. acnes* isolate was obtained as part of approval number Ref53/99 and the *S. lugdunensis* isolate was obtained as part of approval number Ref86/01.

### *Bacterial isolates*

The *P. acnes* strain LED2 was isolated after ultrasound treatment of a retrieved prosthetic hip joint, which was removed at Musgrave Park Hospital, Belfast, Northern Ireland, due to a supposed aseptic joint failure. Bacterial biofilm was also detected in the sonicate fluid by immunofluorescence microscopy (IFM) after labeling with a *P. acnes*-specific antibody as previously described (59). The *S. lugdunensis* strain 010729 was isolated from an intramedullary (IM) nail-fixed tibial fracture at the Royal Victoria Hospital, Belfast, Northern Ireland. Although the fracture had united, the patient reported ankle pain. *S. lugdunensis* was also isolated from the nail plug and locking screws. Bacterial stock cultures were stored at -80°C in 20% (v/v) glycerol using the cryoprotectant bead (Protect™) system. *P. acnes* LED2 was grown anaerobically from stock cultures on Anaerobic Blood Agar (ABA, Oxoid) in a GasPak EZ System with anaerobic sachets (BD Diagnostics, Allschwil, Switzerland). Broth culture of LED2 was performed in proteose peptone yeast (PPY) broth (Oxoid, Basel Switzerland). Immediately before use, 2 mL of L-cysteine (3.75% w/v, Sigma Aldrich, Buchs, Switzerland) and 2 mL of sodium bicarbonate (Sigma Aldrich) were added to 100 mL of the PPY medium to maintain a reducing environment. *S. lugdunensis* 010729 was cultured on tryptic soy agar (TSA, Oxoid) or in tryptic soy broth (TSB, Oxoid) in ambient air at 37°C.

### *Molecular analyses*

Genomic DNA was prepared from single colonies using the Wizard Genomic DNA preparation kit (Promega, Dübendorf, Switzerland) according to the manufacturer`s protocol. The primers used for PCR amplification are listed in Table 1. PCR amplification was carried out using a Eppendorf Thermal Mastercycler gradient (Vaudaux-Eppendorf, Basel, Switzerland) in a total volume of 12.5 µl containing 10x PCR buffer (Invitrogen, Zug, Switzerland), 5 mM dNTP Mix (Promega), 50 pmol of each primer (Microsynth, Balgach, Switzerland), 1 unit of Taq DNA polymerase (Invitrogen) and 10-50 ng template DNA. Products were separated by electrophoresis on 1% (w/v) agarose (Sigma-Aldrich) gels, stained with RedSafe™ Nucleic Acid Staining solution (Intron Biotechnology, Basel, Switzerland) and visualized with UV light and imaged using a GelDoc™ XR image analysis station (BioRad, Reinach, Switzerland). Product sizes were estimated using BenchTop 100 bp and 1 kb DNA ladders (Promega) as molecular size markers. PCR products were purified for sequencing using the PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) following the manufacturer`s protocol.

***Nucleotide sequencing***

Automated sequencing was performed at Microsynth AG, (Balgach, Switzerland) on an Applied Biosystems ABI3730xl Sequence Analyser 5.2 using the ABI Big Dye system V3.1. Sequences were compared with known sequences using BLAST (http://blast.ncbi.nlm.nih.gov).

***In vitro adhesion and biofilm formation***

*In vitro* adhesion of the bacteria to a range of orthopedic metals was measured using the pre-operative contamination model of Rochford *et al.* (45). The metals used were electropolished stainless steel (SS), standard micro-rough titanium-aluminum-niobium (NS) and electropolished titanium-aluminum-niobium (NE). Adhesion studies were performed in phosphate buffered saline (PBS) for *S. lugdunensis* and PBS supplemented with L-cysteine (0.05% v/v) for *P. acnes*. Briefly, the bacterial suspension was adjusted to an optical density of 0.5 at 600 nm and diluted 10-fold to give a 1 L suspension at an approximate density of $1\times10^7$ colony forming units (CFU) per ml, this value was confirmed for each experiment by total viable counts (TVCs). Triplicate 13 mm discs of test materials were placed into the adhesion chamber and the liquid was stirred at 125 rpm for 2.5 h at 37°C to measure initial adhesion. The sample discs were retrieved from the chamber and placed in sterile glass bottles containing 5 ml of PBS. The discs were then ultrasonicated for 3 min using an ultrasonic water bath (Bandelin, Germany) operating at 40 kHz, followed by vortex mixing for 20 s to remove the adherent bacteria. TVCs of the removed bacteria were carried out by serial dilution and plating on

blood agar (BA, Oxoid) or ABA, which were incubated aerobically for 2d or anaerobically for 14 d for the detection of *P. acnes* or *S. lugdunensis*, respectively. Biofilm formation was measured by the method of Stepanovic *et al.* (53). For *S. lugdunensis* isolate 010729, biofilm formation was measured after 24 h of incubation in TSB supplemented with 1 % w/v glucose (Sigma-Aldrich), as recommended by the Stapanovic protocol to ensure reproducible results for staphylococci. For LED2, biofilm formation was measured after 7 d of incubation in PPY broth supplemented with L-cysteine and sodium bicarbonate as described above.

***In vivo animal model***

Custom-made IM nails composed of SS (ISO 5832/1), and 2.5 mm in diameter and 85 mm in length, were used. Prior to implantation, all IM nails were washed twice in 70% (v/v) ethanol followed by two periods of sonication in deionized water using an ultrasonic water bath operating at 40 Hz for 15 min. Each nail was then packaged individually and steam sterilized at 121°C for 20 min. Skeletally mature, specific pathogen-free female New Zealand White rabbits (Charles River, Sulzfeld, Germany) were used in this study. All rabbits were assessed by a veterinarian and determined to be healthy prior to inclusion in the study.

Prior to surgery, each rabbit was screened to determine whether the IM nail would be accommodated within the tibia. Under general anesthesia and using aseptic surgical techniques, the patellar ligament of the left tibia was divided and the anterior part of the tibia plateau exposed. The medullary cavity was opened with a 3.5 mm drill bit and evacuated with a 3.0 mm diameter suction device. The distal two thirds of the medullary canal were inoculated with 50 µL of bacterial suspension in ¼ strength Ringers solution (25% v/v, QSRS) using a catheter. Bacterial inocula were added to the implant site within 15 min of preparation. The implant was immediately inserted without hammering and the insertion site sealed with a water-soluble alkylene co-polymer hemostasis material (Ostene, CEREMED, Inc. California, USA). The patellar ligament and skin was closed in a routine manner. To minimise variability, the same surgeon operated on all rabbits.

The *P. acnes* inoculum for the animal study was prepared by sub-culturing a single colony of freshly grown LED2 from ABA into 50 ml PPY broth. The culture was incubated anaerobically for 16 h at 37°C. Immediately prior to surgery, an aliquot was taken and centrifuged at 2500 rpm for 10 min and the resulting pellet re-suspended in pre-reduced QSRS containing the reducing agent L-cysteine (0.05% v/v). The inoculum density was adjusted to a target of 3 x $10^7$ CFU/50 µl based on an optical

density of 0.5 at 600 nm. For the *S. lugdunensis* inoculum, one colony was taken from a young (<24 h) TSA culture and inoculated into 20 ml pre-warmed TSB. The culture was incubated for 2 h on a shaking water bath at 37°C and 100 rpm. Prior to each surgery, an aliquot was taken and centrifuged at 2500 rpm for 10 min, re-suspended in QSRS and similarly adjusted to a target density of 0.6 at 600 nm, approximately 3 x $10^7$ CFU/50 µl.

Quantitative culture of each inoculum was performed immediately after preparation to determine the exact number of bacteria given to each animal. Serial ten-fold dilutions of the bacterial culture were performed in QSRS and plated onto BA for 24 h (*S. lugdunensis*) or ABA plates for 14 d (*P. acnes*).

***Observation and euthanasia***

Upon completion of the surgical procedure, baseline radiographs were taken and the rabbits were returned to their individual hutches and monitored for 4 weeks. Each animal was monitored continuously throughout the observation period for signs of systemic infection. No antibiotics were administered to any animal during this study. Daily evaluation of attitude, appetite and surgical incisions was carried out whilst weight and body temperature were recorded weekly. Blood samples were collected preoperatively and weekly thereafter for white blood cell (WBC) count (Vet ABC, Scil animal care, Viernheim, Germany). Animal exclusion criteria were set at a weight loss exceeding 10% of the initial body weight within two weeks, local infection with severe lameness, persistent swelling and discharge, or signs of systemic infection such as fever, depression and anorexia. After 28 d the animals were humanely euthanized. Pilot surgeries were performed, whereby similar implants were placed, without any inoculation, in two animals. Weight, WBC and histology images of these un-inoculated animals are included for comparison.

***Post-infection bacteriology***

All rabbits receiving an inoculum of either *P. acnes* or *S. lugdunensis* were processed for bacteriology. After euthanasia, the skin was removed from the left leg and wiped down with 70% (v/v) ethanol. Swabs were taken from the tibial plateau at the point of insertion of the nail and streaked across BA plates. The tibia was then cut using an oscillating saw to expose the most distal end of the nail. The nail was removed through the distal end of the tibia and submerged in 6 ml of sterile QSRS, gently agitated for 20 s to remove loosely adherent bacteria, and then transferred to a second bottle of sterile QSRS. The nail was then vortexed for 30 s and subsequently placed in a sonicating water bath operating at a frequency of 40 kHz for 3 min before a final vortex for 30 s. The sonicates were

immediately serially diluted in sterile QSRS and 200 µl aliquots spread onto TSA, BA and ABA plates for quantitative bacterial culture. The tibia was then cut just below the most proximal point previously occupied by the IM nail and the proximal portion fixed for histological processing as described below. A 2 cm section was also removed from the distal end of the tibia for histology. The remainder of the bone that previously surrounded the IM nail was then homogenized in 15 ml QSRS with or without L-cysteine as appropriate, using a Polytron PT3100 homogenizer (Kinematica AG, Switzerland). Serially ten-fold diluted and duplicate 200 µl aliquots of the bone homogenate were added to TSA, BA and ABA plates for incubation. All TSA and BA plates were incubated at 37 °C aerobically, the ABA plates incubated anaerobically, and results recorded at 24 h and re-examined after 48 h for *S. lugdunensis* and 14 d for *P. acnes* inoculated rabbits. In both groups, aerobic and anaerobic culture was performed to ensure no secondary infection was present. The lower limit of detection of the bone samples was 75 CFU per bone fragment and 30 CFU for the IM nail. To be considered infected, either the bone or the LCP was required to yield at least five colonies on both agar plates of the undiluted suspension. All bacterial growth was identified using the Remel Rapid ANA II and the RapID STAPH PLUS System test kits performed according to manufacturer's protocols (Remel, Switzerland).

***Histology***

The proximal and distal tibiae were fixed in 70% (v/v) methanol and subsequently decalcified with EDTA. Decalcified blocks were trimmed to include the entry point of the nail and the nail cavity, and embedded in paraffin. Six-micrometer sections were cut and stained with Haematoxylin and Eosin for histomorphological scoring. Adjacent or nearby sections were stained with a modified Brown and Brenn stain to visualize bacteria.

The regions of interest for histological scoring were the articular surface, entry site and tract of the nail, the medullary canal, cortical bone and periosteum. Representative sections were graded by a histopathologist, who was blind to the rabbit treatments, for severity and characterization of the infection.

Electron microscopy was performed on representative sections to identify the location of the bacteria within the tissues. Sections were deparaffinized, sputter coated with Gold Palladium and then imaged using a Hitachi S4700 scanning electron microscope (SEM). Images were taken in the secondary electron mode with an accelerating voltage of 5 kV.

**RESULTS**

***Strain characterization and adhesion properties***

*P. acnes* strain LED2 belongs to the type IB sequence type (ST) 5 lineage [clonal complex (CC) 5], based on multilocus sequence typing (MLST) analysis of six core housekeeping genes and two 'putative virulence' genes (26-28). It is hemolytic on horse and sheep ABA, has a demonstrable co-hemolytic CAMP reaction and expresses an abundance of the CAMP factor 1 protein as detected by SDS-PAGE and immunoblotting (61). In keeping with its identification as a type IB strain, it does not react with monoclonal antibodies specific for the phase variable dermatan sulphate-binding adhesins present on the surface of type IA and IC isolates (25). *S. lugdunensis* 010729 is positive for the *vwbl* gene, which encodes the Von Willebrand factor, and fibronectin-binding proteins encoded by *fbn*, *fbl* and *fbpA* genes. Genes encoding β-hemolysin and hemolytic peptides were also detected by PCR amplification. The intercellular adhesion gene *icaA* was also present.

In the bacterial adhesion assay, *S. lugdunensis* 010729 adhered to all of the metals tested in similar amounts (ANOVA, p=0.790), though the variation amongst the results was high (Figure 1). The number of viable *P. acnes* retrieved from the materials was less than *S. lugdunensis,* although initial inocula were equivalent between groups. In addition, there were significantly fewer *P. acnes* adhering to NE compared to SS and NS (ANOVA p=0.050). The ability to form biofilm *in vitro* was determined for LED2 and 010729 using the crystal violet staining method and classification scheme of Stepanovic *et al.* (53). Under these conditions, *S. lugdunensis* produced a moderate biofilm within 24 h with an optical density (A600) of 0.12 ± 0.01 SD, whilst *P. acnes* produced a moderate biofilm with A600 of 0.10 ± 0.01 SD at day 7; however, only weak biofilm (A600 below 0.05) formation was identified prior to day 7.

***Surgery and observation***

Postoperative radiographs showed good placement of the IM nail without any observable fractures. All rabbits tolerated the surgical procedure and no animals were excluded from the study due to perioperative complications or postoperative sepsis. Post-euthanasia radiographs showed there was no migration of the implant, and no radiographic signs of severe osteolysis were observed (Figure 2). Table 2 shows weight and WBC values for *P. acnes* and *S. lugdunensis*-infected animals and un-inoculated rabbits throughout the study period. Un-inoculated rabbits displayed a minor peak in WBC and weight loss in the first post-operative week, both of which had returned to pre-operative values prior to completion of the observation period, with overall weight gain observed by completion of the

study. The *P. acnes*-infected rabbits displayed minor weight loss in the first postoperative days followed by full recovery and some weight gain, though less than un-inoculated animals. The WBC count also showed a minor spike in the first weeks, which was greater and more persistent than that observed in un-inoculated animals. However, WBC had returned to normal after the four-week observation period. The *S. lugdunensis*-infected rabbits displayed, on average, a greater weight loss in the first two weeks and by the end of the study had not gained any weight. The WBC count also showed a more pronounced and persistent elevation than that displayed by the *P. acnes*-infected animals, although baseline WBC count was greater in the *S. lugdunensis* group.

***Bacteriological outcomes***

The average intraoperative bacterial inoculum given to the rabbits that received *P acnes* LED2 was 1.67 x $10^7$ CFU. Upon completion of the observation period, only 1/6 *P. acnes* inoculated animals were culture positive from the joint swab. Bone cultures were positive for all six *P. acnes*-inoculated rabbits. Quantitative bacteriology after sonication of the implant showed that all six *P. acnes* inoculated animals were positive for biofilm on the IM nail. The TVC of biofilm (sonication fluid and loosely adherent biofilm) and bone cultures (per gram) is shown in Figure 3. On average, the total CFU count cultured from the culture-positive bone samples and implants was 2.6 x$10^5$ CFU, and 3.3 x$10^6$ CFU, respectively. In all cases, the microorganism cultured from the bone or the implants of the LED2 inoculated animals was confirmed as *P. acnes* using Remel rapid ID diagnostic kits. IFM analysis with a monoclonal antibody (QUBPa3) (29) to *P. acnes* also confirmed the presence of the bacterium in sonicate samples (data not shown). No other bacterial species, based on colony morphology, were identified in any of the samples from these rabbits.

The average intraoperative bacterial inoculum given to the rabbits that received *S. lugdunensis* 010729 was 2.49 x $10^7$ CFU. At completion of the four-week observation period, 4/6 *S. lugdunensis*-inoculated rabbits were culture positive from the joint space swab. Bone cultures were also positive for the same rabbits, and negative in the remaining two rabbits. No bacteria were isolated from animals recorded as culture-negative. Quantitative bacteriology after sonication of the implant showed that only 2/6 rabbits receiving the *S. lugdunensis* inoculum were positive for biofilm on the IM nail, both of whom were also culture positive from the bone. The TVC of biofilm (sonication fluid and loosely adherent biofilm) and bone cultures (per gram) of each animal is shown in Figure 4. On average, the total CFU counts cultured from the positive bone samples and implants was 5.1 x$10^6$ CFU and 1.3x$10^6$

CFU, respectively. In all cases, the microorganism cultured from the bone or the implant of the *S. lugdunensis*-inoculated animals was confirmed as *S. lugdunensis* using the REMEL diagnostic kit. Sequence analysis of the 16S rDNA PCR fragment revealed 100% identity to the sequence of *S. lugdunensis* present in GenBank under Accession number N920143. As with *P. acnes*, IFM with a monoclonal antibody (QUBSe1) to staphylococci also confirmed the presence of the bacterium in sonicate samples (data not shown). No other bacterial species, based on colony morphology, were identified in any of the samples from these rabbits. All samples from the un-inoculated rabbits were negative from the joint swab, the bone samples and the sonicated nail sample.

***Histology***

Characteristic features of osteomyelitis, such as micro- and macro-abscesses, and increased presence of neutrophils and other inflammatory cells were observed to varying degrees in all six *P. acnes*-infected rabbits (summarized in Table 3). Focal inflammatory changes were seen in the sub-articular region, however, the presence of fibrocartilage indicated the ulcerations were healing and no sub-articular macro-abscesses were identified (Figure 5). A higher-grade osteomyelitis was observed adjacent to the implant, where diffuse inflammation is seen with some preservation of the microarchitecture (Figure 6). These pathologies are not observed in this model system as a result of implantation of the IM alone (Figure 5 and 6). After Brown and Brenn staining, all specimens revealed the presence of bacteria, which primarily localized in small clusters located between adipocytes in the distal medullary cavity, which was confirmed by SEM imaging (Figure 7).

The histological features of the *S. lugdunensis* infection were consistent with pyogenic osteomyelitis and septic arthritis, as summarized in Table 3. Focal inflammatory changes were seen in all animals with either micro- or macro-abscesses in the sub-articular region (Figure 5 and 6). There was evidence of septic arthritis in all six rabbits, with sub-articular abscesses communicating with the adjoining joint in four of the animals. These pathologies are not observed in this model system as a result of implantation of the IM alone (Figure 5 and 6). After Brown and Brenn staining, bacteria could be seen in all specimens examined (Figure 7), including the two culture-negative animals. Bacteria were seen on the edges of the sub-articular abscesses, whilst none or few were identified within the abscesses themselves. Small clusters were also located between adipocytes in the medullary cavity adjacent to activated erythro- and myelo-poetic cells, indicative of an early osteomyelitis. Some bacterial cells also appeared to have been phagocytozed by inflammatory cells in the bone marrow,

although viability of these bacteria was not determined (shown in Figure 7). SEM showed scattered presence of *S. lugdunensis* throughout the tissue (Figure 7), although an intracellular localization of *S. lugdunensis* could not be confirmed by SEM.

## DISCUSSION

Historically, microorganisms such as *P. acnes* and CoNS have been considered contaminants from the skin rather than causative agents of osteomyelitis (9,11,39). Reports from the Patrick laboratory at Queen's University Belfast in the late 1990s (59,60) revealed the isolation and detection of these microbes in significant numbers from 'aseptically' loosened failed implants by the use of ultrasound to dislodge adherent biofilm. This data suggested that these apparently 'low virulence' commensal microorganisms might in fact be important pathogens capable of causing significant numbers of implant-related bone infections, leading to osteolysis and implant failure. Subsequent studies have supported these findings (6,22,42,43,57) and further work at QUB also provided evidence of colonization of bone adjacent to the implant (40). Despite this, the question of whether these bacteria are passive by-standers or drivers of prosthetic joint failure has been an on-going subject of debate, with wear debris arising from implant materials still considered a major driver of implant failure (37).

While a large amount of research has been carried out on understanding the pathogenic nature of *S. aureus* and *S. epidermidis,* there is only limited information available for *P. acnes and S. lugdunensis*, and even fewer studies focusing upon preclinical *in vivo* models of infection (4,12,21,46). This lack of study also extends to the appropriateness of antimicrobial therapies for these microorganisms, the serological response to their infection, and the role of microbial virulence factors on the progression of infection. To address this issue, we established an experimental implant-related osteomyelitis model, based upon one described for *S. aureus* (32), for the study of *P. acnes* and *S. lugdunensis*-related infections. The model incorporates implanted IM nails which, as with all indwelling biomaterials, provide enhanced colonization opportunities for professional pathogens and opportunistic pathogens alike. For our study we used a strain of *P. acnes* that was isolated from sonicate prepared from a failed prosthetic hip joint. This isolate had been previously characterized by MLST, which revealed that it belonged to the ST5 lineage within the type IB phylogenetic grouping (26). Isolates from this phylogroup are associated with healthy skin, and rarely recovered from acne vulgaris lesions. They have, however, been associated with soft tissue and medical device-related infections, although their exact clinical importance in these cases has remained unclear (26,28). To date, a total of 13 distinct

type IB STs have been described in the *P. acnes* MLST database (http://pubmlst.org/pacnes/) based on the analysis of 69 isolates, of which 11 belong to a single CC with ST5 as the founder genotype (CC5) (bootstrap value of 99%), while two isolates are singletons (ST12, ST51). As expected for a founding genotype, the ST5 clonal lineage is highly prevalent in the human population and is globally disseminated. *P. acnes* contains an expanded family of five CAMP factor homologues that appear to have arisen primarily as a result of horizontal gene transfer (HGT) as judged on previously described co-localization and sequence similarity criteria (28). Previous studies with five type IB *P. acnes* isolates (all subsequently identified as the ST5 lineage by MLST) demonstrated abundant production of the CAMP factor 1 protein when compared to strains from the type IA division; secretion of large quantities of CAMP 1 were also observed with isolates from the large type II clade (26,61). Currently, the exact role played by CAMP 1 and the other CAMP factor homologues is unclear, but they may play an important role in the survival of *P. acnes* within the human host as well as contributing to a pathogenic lifestyle (28). Recently, an MLST scheme and database for *S. lugdunensis* was described based on the analysis of seven core housekeeping genes (8). A clonal population structure with limited sequence diversity was revealed, and isolates recovered from hematogenic infections (blood or osteoarticular isolates) or from skin and soft tissue infections were not found to cluster in separate lineages. Currently we do not have MLST data for *S. lugdunensis* 010729, but we are now analyzing the strain by whole genome sequencing which will facilitate immediate assignment of a ST and CC when data for each of the MLST loci is complete.

The experimental model used here requires the direct, artificial inoculation of bacteria into the surgical field, which it must be noted will exceed the number of bacteria likely to contaminate a surgical wound during a primary, elective procedure. However, if a failed prosthetic joint with a mis-diagnosis of aseptic loosening is removed, and a new sterile device placed in the underlying infected site it is very possible that higher numbers of bacteria will be present, particularly if adjacent bone has been colonized. The bacterial inoculum we used ($3 \times 10^7$ CFU) is well within the range of inocula previously applied in other experimental rabbit models of staphylococcal implant-related osteomyelitis (for example $3 \times 10^6$ to $2 \times 10^8$ CFU) (1,32,36). Upon completion of the study, the total CFU counts recovered from both *P. acnes* and *S. lugdunensis*-infected rabbits were quite variable, although the majority of culture positive samples were between $1 \times 10^6$-to-$1 \times 10^7$ CFU for *P. acnes* and $1 \times 10^3$-to-$1 \times 10^7$ CFU for *S. lugdunensis*. The total numbers of bacteria quantitatively cultured from this study are similar to the numbers of *S. aureus* cultured from the previous model on which our study is based (32).

The lower recovery of *S. lugdunensis*, in particular the lack of consistent biofilm, is an interesting finding further discussed below.

The general histopathological features of osteomyelitis observed in this model included the presence of diffuse, focal and multifocal inflammation characterized by infiltration of the medullary canal by neutrophils and the presence of sub-articular and peri-implant abscesses with surrounding fibrosis. A histopathological scoring system, based upon similar studies using *S. aureus* (20,41,52), was developed to specifically focus upon the features of implant-related osteomyelitis as it occurs after intramedullary nailing. This scoring system was then used to determine whether there were differences in the characteristics of any infection caused by these two species of bacteria. In operated but un-inoculated animals, the entry point of the nail healed uneventfully and the medullary area displayed a physiological appearance, lacking in increased cellularity or significant fibrosis. A comparatively severe histopathology, characterized by macro-abscess formation and septic arthritis, was observed after infection with *S. lugdunensis*. Although *S. aureus*-infected animals were not compared directly in this implant model, the histopathology of the *S. lugdunensis* inoculated animals was comparable to that seen in a similar animal model using *S. aureus* (41).This is consistent with the association of *S. lugdunensis* with a clinical course more similar in severity to a typical *S. aureus* infection than to other CoNS (13). A complete genome sequence of *S. lugdunensis* did not reveal the well-recognized virulence determinants that enable *S. aureus* to cause acute infection, but different strains of *S. lugdunensis* may carry other putative virulence determinant genes gained as a result of HGT (13,17). Interestingly, the *S. lugdunensis* infection was not significantly associated with biofilm formation, as biofilm was detected by culture on the IM nail of only 2/6 animals. This is despite *in vitro* biofilm formation by this *S. lugdunensis* isolate on a range of implant materials. The *S. lugdunensis* bacterium was, however, observed in all histological sections after 28 days *in vivo*. The histological analysis revealed that the *S. lugdunensis* was predominantly found adjacent to abscesses, and in many cases appeared intracellularly within phagocytic cells in the abscess tissue. The viability of these intracellular bacteria is not known, but intracellular survival is a feature associated with *S. aureus* and *S. epidermidis* and is entirely in keeping with the intracellular survival of other CoNS (5). The intracellular location of *S. lugdunensis* would be a potential explanation for the culture-negative animals observed in this study. Non-culture-based detection of staphylococci has also previously been described for culture-negative retrieved failed prostheses and biopsies from human patients with long bone non-union of fractures (38). The culture-negative animals may also be due to an unavoidable

sampling error, leading to bacterial presence in the bone sample retained for histology, yet absent from the biopsies retained for bacterial culture. What is clear, however, is that the histopathology of the culture-negative *S. lugdunensis* inoculated animals indicates an active infection in these animals. To definitively characterize the natural progression of the infection in this model, and whether this culture-negative finding indicates a subsidence of the infection, requires further study.

In the *in vivo* model, *P. acnes* causes an infection characterized by biofilm formation and localized inflammation adjacent to the biofilm and implant. According to the histological scoring, *P. acnes* did not cause septic arthritis and the entry wound from the insertion of the IM nail was found to be healing with fibro-cartilaginous tissue formation. The histological scoring of the medullary area adjacent to the implant and the biofilm was greater than the sub-articular region, and even greater in severity than the *S. lugdunensis* data. These pathologies are not observed with implantation of the IM alone (Figure 5 and 6). The *P. acnes* micro-colonies were observed primarily adjacent to the adipocytes within the medullary cavity, some of which also showed regions with a high-grade osteomyelitis. SEM analysis confirmed micro-colony formation (Figure 7) in the intercellular regions in the bone marrow, and there was no evidence of intracellular localization of *P. acnes* by SEM or light microscopy. The clinical signs, such as weight loss and white cell count were less severe in the *P. acnes*-infected animals in comparison to those infected with *S. lugdunensis*. Un-inoculated animals gained more weight than *P. acnes*-infected animals, which is supportive of the view that *P. acnes* causes chronic low grade infections, characterized by subtle clinical signs and symptoms and a significantly different histopathology from staphylococcal osteomyelitis.

Overall, both the *in vitro* and *in vivo* evaluations indicated that *P. acnes* differs in many respects to *S. lugdunensis*. Our *in vitro* study revealed that *P. acnes* adhered relatively poorly to a range of implant materials when compared to *S. lugdunensis*. *In vitro* formation of biofilm and adhesion to orthopedic materials has previously been shown for *P. acnes* (3,18,44,58,61), although correlation between *in vitro* and *in vivo* studies is lacking. Our isolate, LED2, displayed weak biofilm formation *in vitro,* although this is clearly not representative of the clinical situation, or even for our experimental infection. Our *in vivo* data revealed that *P. acnes* formed biofilm in all the animals examined; in contrast, *S. lugdunensis* appeared to have reduced ability under these conditions. The relationship between biofilm formation and osteomyelitis therefore requires further investigation, particularly in the case of *S. lugdunensis.* Of high importance is the observation that a more severe medullary

431 osteomyelitis was observed in *P. acnes*-infected animals, when compared to those infected with *S.*
432 *lugdunensis*. The most severe grade of intramedullary osteomyelitis, Grade 5, was observed in three
433 rabbits infected with *P. acnes*, but in none infected with *S lugdunensis*. The model used is a non-
434 loaded IM nail without any interlocking bolts. As such, there is no risk of any wear-induced particles or
435 fretting corrosion and no such particles were observed in any histological section. Furthermore,
436 osteomyelitis was not observed where the IM was implanted in the absence of either *S. lugdunensis* or
437 *P. acnes*. This indicates that *P. acnes* can cause osteomyelitis in the absence of implant material wear
438 debris and in the absence of overt clinical signs of infection. With regard to *S. lugdunensis*, the model
439 confirms that this is a potentially pathogenic CoNS, and that biofilm formation is not a prerequisite for
440 infection.

441 These results have important implications in relation to distinguishing between true aseptic joint
442 loosening, driven solely by, for example, wear particle mediated inflammation, and chronic infection-
443 driven loosening which may also be combined with wear debris involvement. These findings may also
444 have wider implications in relation to the involvement of such bacteria in other conditions such as
445 dental infection (34) and synovitis-acnes-pustulosis-hyperostosis-osteitis (SAPHO) syndrome (7,49). It
446 would be interesting to follow the progression of both infections histologically over a greater time
447 period, and as it becomes more chronic in nature. It would also be valuable to investigate the effect of
448 these pathogens on pseudoarthrosis or delayed healing; however, currently there is no standardized
449 rabbit model of implant related osteomyelitis with a fracture. Nevertheless, the model showed that the
450 progression of implant related osteomyelitis follows a bacterial species-specific course and highlights
451 the potential of *P. acnes* and *S. lugdunensis* to cause significant implant related osteomyelitis.

452 Based on the study, we conclude that both *P. acnes* and *S. lugdunensis* are pathogens capable of
453 producing osteomyelitis in a rabbit model, but exhibit different pathologies. The infection caused by the
454 S. *lugdunensis* was in keeping with a classically virulent microbe, with macroabscess formation and
455 failure to heal entry wounds, but only a maximum Grade 4 intramedullary osteomyelitis was observed
456 in a single rabbit, with the remaining rabbits experiencing a lower grade osteomyelitis. Interestingly,
457 the *S. lugdunensis* infection was not always characterized by biofilm formation, even when bacteria
458 were present in the adjacent bone. This result may have important clinical implications with regard to
459 the location and extent of tissue debridement required for implant revision/removal, which commonly
460 centers only on implant removal. In contrast, the infection caused by *P. acnes* was characterized by

healing of the entry wound and low morbidity for the animals; however, there was extensive biofilm formation and, most significantly, evidence of Grade 5 intramedullary osteomyelitis. This occurred in the absence of implant material wear debris. This experimental study therefore provides strong evidence for a potential key role of *P. acnes* in prosthetic joint infection and fracture non-union, in the absence of signs of classical infection and patient morbidity. *P. acnes* should therefore no longer be dismissed as an insignificant pathogen in the setting of failed retrieved implants; clinical diagnostic practice should be tailored to enable the efficient detection of *P. acnes*. Without this there is the risk of an incorrect diagnosis of aseptic loosening and subsequent patient treatment may be misinformed.

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**List of Figures**

Figure 1. Total viable counts of bacteria retrieved from Stainless Steel (SS), Electropolished Titanium-Aluminum-Niobium (NE) and Standard micro-rough Titanium-Aluminum-Niobium (NS) after incubation *in vitro* for 2.5 h with *P. acnes* LED2 and *S. lugdunensis* 010729.

Figure 2. Representative postoperative and postmortem radiographs of rabbit tibiae. (A) postoperative radiograph of a rabbit that received a *P. acnes* inoculum; (B) a postmortem radiograph of the same animal after 28 d (C). Similar post-operative radiograph for a rabbit receiving a *S. lugdunensis* inoculum (D) a post mortem radiograph again from the same animal taken after 28 d. Note: no radiographically evident signs of infection were observed for either bacterial species.

Figure 3. Quantitative culture of *P. acnes* LED2 from the bone and implant of each rabbit

Figure 4. Quantitative culture of *S. lugdunensis* 010729 from the bone and implant of each rabbit

Figure 5 Micrographs illustrating histological analysis of the point of entry of the nail and subarticular region for (A) uninfected, (B) *P. acnes* LED2-infected and (C) *S. lugdunensis* 010729-infected rabbits. In the uninfected animals (A), the entry point has healed completely and no sub-articular abscesses are observed. In the *P. acnes* infected animals (B), healing of the point of entry of the nail is also seen and no signs of sub-articular abscess formation were observed. Increased inflammatory cells were present deep in the medullary cavity adjacent to the implant site (arrow). In the *S. lugdunensis*-infected animals (C), macroabscesses were observed just below the articular surface (arrow) (grade 4). Haematoxylin and Eosin staining.

Figure 6. Micrographs illustrating histological analysis of the medullary region of (A) uninfected, (B) *P. acnes* LED2-infected and (C) *S. lugdunensis* 010729-infected rabbits. In the uninfected animals (A), the medullary region displays normal physiology with abundant univacuolar adipocytes and the lack of cellular infiltration. In the *P. acnes* infected animals (B), a diffuse, marked increase in cellularity indicative of grade 3 osteomyelitis is seen (arrow). In *S. lugdunensis*-infected animals, (C) focal inflammatory changes, such as microabscesses are seen in the medullary region with a marked decrease in adipocytes (arrow). Haematoxylin and Eosin staining.

Figure 7 Micrograph illustrating the localization of bacteria within infected animals. *S. lugdunensis* was primarily associated with abscesses (A) and an intracellular localization of *S. lugdunensis* was also observed (Modified Brown and Brenn staining). (B) Scanning electron micrograph of bacterial cells in medullary area (arrow). (C) *P. acnes* were arranged in clusters (arrow) adjacent to adipocytes in the medullary cavity and (D) scanning electron micrograph of bacterial cells in the medullary area. Note the pleomorphic rod morphology characteristic of *P. acnes* (arrow).

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737 **Table 1 Primers used in this study**

| Target | Gene/ locus-tag | Primer name | Sequence | Reference |
|---|---|---|---|---|
| 16S rDNA | | fD1 | CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG | (64) |
| | | rP2 | CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT | (64) |
| Von Willebrand factor | *vwbl* | stlu_vwbl_F | TGGCGGGATGATTTGGACGGG | (55) |
| | | stlu_vwbl_R | TCGCCTTCTTGCCCTGATGGT | (55) |
| Fibronectin binding | *fbn* | F1 | GGTAATCAGTCATTCGAG | (31) |
| | | R1 | TGGCACACTGTCGAAGTC | (31) |
| Fibrinogen binding | *fbl* | fbl_check_F | CGTATTATCCCAAGTAGCAACC | (55) |
| | | fbl_check_R | CTTCATCGATTGTCCCAGTAGC | (55) |
| Fibrinogen binding | *fbpA* | FbpA_F | GAGATTACTGGACAACAAACG | (55) |
| | | FbpA_R | GTATTGTGACGTCGTTTCCTG | (55) |
| Hemolytic petides | *slush* | slush_donvito_F | TTTCGTCTTTGCACACACATTTCCA | (55) |
| | | slush_donvito_R | ACAGCACAAAGCCTTAACTATCTCA | (55) |
| Tannase A | *tanA* | tanA-F | AGCATGGGCAATAACAGCAGTAA | (35) |
| | | tanA-R | GCTGCGCCAATTTGTTCTAAATAT | (35), |
| Beta-hemolysin | SLGD_00006 | betahemolysin_F | TGGTCAAGGTACAGAAGGTTGGCA | (55) |
| | | betahemolysin_R | TATCCCAACTATACGCGTTGCCCT | (55) |
| Intercellular adhesion gene A | *icaA* | ICAF | GATGGAAGTTCTGATAATAC | (48) |
| | | ICAR | CCTCTGTCTGGGCTTGACC | (48) |

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741 **Table 2 Change in body weight and White Cell count over time. Mean ± Standard deviation (range)**

| | Pre-operative | 3 days | 7 days | 14 days | 21 days | 28 days |
|---|---|---|---|---|---|---|
| **Uninoculated** | | | | | | |
| Weight [1] change(Kg) | 0.00 | 0.044 ± 0.06 | -0.06 ± 0.05 | 0.12 ± 0.08 | 0.12 ± 0.09 | 0.32 ± 0.08 |
| WBC ($x10^3$/mm3) | 5.11 ± 1.16 | 6.95 ± 1.56 | 7.12 ± 1.32 | 5.77 ± 1.01 | 5.82 ± 1.43 | 6.0 ± 1.22 |
| ***P. acnes* LED2** | | | | | | |
| Weight change (Kg) | 0.00 | -0.15 ± 0.11 (-0.34/ -0.01) | -0.11 ± 0.10 (-0.24/ -0.01) | 0.15 ± 0.09 (+0.33/+0.08) | -0.07 ± 0.111 (0.09/ -0.22) | 0.17 ± 0.107 (0.30/ -0.01) |
| WBC ($x10^3$/mm3) | 3.82 ± 0.67 (5.14 / 3.24) | 8.10 ± 1.40 (10.66/-6.8) | 8.16 ± 1.16 (9.78 – 7.01) | 7.07 ± 1.58 (10.18 /5.77) | 6.83 ± 0.15 (6.93/6.72) | 3.86 ± 0.79 (4.42/3.29) |
| ***S. lugdunensis* 010729** | | | | | | |
| Weight change (Kg) | 0.00 | -0.27 ±0.11 (-0.43/-0.15) | -0.23 ±0.03 (-0.26/ -0.17) | -0.08 ± .097 (-0.26/ -0.03) | -0.08 ± 0.05 (-0.17/ -0.03) | -0.01 ± 0.05 (-0.08/+0.06) |
| WBC ($x10^3$/mm3) | 4.70 ± 1.22 (6.49/3.43) | 9.26 ± 3.51 (16.27/ 7.06) | 10.56 ± 1.98 (12.21/7.1) | 8.83 ± 1.49 (11.61/7.36) | 7.42 ± 1.15 (8.23/6.6) | 4.91 ± 1.24 (5.79/4.03) |

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743 [1] **Change in body weight from Preoperative weight.**

744 **Table 3 Summary of the histological findings associated with *P. acnes* LED2 and *S. lugdunensis* 010729 infection**

| Location | Histological feature | Grade /score | *Staphylococcus lugdunensis* 010729 | | *Propionibacterium acnes* LED2 | |
|---|---|---|---|---|---|---|
| **Articular Cartilage** | Presence of ulceration | yes / no | 4/6 | | 1/6 | |
| | Presence of fibrocartilage indicating healed ulceration | yes / no | 1/6 | | 1/6 | |
| | Presence of inflammatory cells or bacteria in the ligament-indicating septic arthritis | yes / no | 6/6 | | 1/6 | |
| **Sub-articular Region, Entry tract and Medullary cavity** | Diffuse increase in the cellularity of the marrow with near normal preservation of the marrow architecture (fat cells and precursors) | Grade 1 myelitis | **Sub-articular** | **Medullary** | **Sub-articular** | **Medullary** |
| | | | 1 | 2 | 2 | |
| | Presence of micro-abscesses up to 5 in number with or without a diffuse increase in cellularity similar to Grade 1 | Grade 2 myelitis | 2 | | 2 | 1 |
| | Presence of micro-abscesses more than 5 in number with or without a diffuse increase in cellularity similar to Grade 1 | Grade 3 myelitis | 2 | 3 | 2 | 1 |
| | Presence of macro-abscesses defined as collection of neutrophils with a fibrous capsule OR a localised gross increase in cellularity | Grade 4 myelitis | 1 | 1 | | 1 |
| | Diffuse gross increase in neutrophils and inflammatory cells in the entire medullary canal inclusive of the periphery and centre | Grade 5 myelitis | | | | 3 |
| | Presence of Necrotic bone or sequestrum | yes / no | 1/6 | 2/6 | 0/6 | 0/6 |
| | Presence of fibrosis | yes / no | 6/6 | 6/6 | 6/6 | 6/6 |
| **Cortical bone** | Increase in the size of the lacunae with neutrophils | yes / no | 2/6 | | 2/6 | |
| | Presence of hyaline degeneration of vessels | yes / no | 0/6 | | 0/6 | |
| | Presence of vascular neutrophilia | yes / no | 3/6 | | 1/6 | |
| | presence of empty lacunae | yes / no | 5/6 | | 1/6 | |
| | Fibrosis | yes / no | 6/6 | | 4/6 | |
| | Presence of Necrotic bone or sequestrum | yes / no | 3/6 | | 0/6 | |
| | Inflammatory cells under the periosteum/ subperiosteal abscess | yes / no | 2/6 | | 3/6 | |

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